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# **MOLECULAR MECHANISMS IN IDIOPATHIC INFLAMMATORY MYOPATHIES**

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## MOLECULAR MECHANISMS IN IDIOPATHIC INFLAMMATORY MYOPATHIES

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# ABSTRACT

**Background:** Myositis is a group of rare autoimmune diseases. Muscle weakness and fatigue are the dominant symptoms and inflammation with T cells and macrophages is a characteristic finding in muscle tissue. Currently high-dose and long-term glucocorticoids is still the most important treatment but with limited efficacy and worrisome side effects. Therefore, investigations regarding inflammatory mediators and their roles in myositis pathogenesis are important in order to develop new therapies.

**Methods:** In order to investigate the roles of different inflammatory mediators in patients with myositis, biological samples were investigated from clinically well-characterized patients in different phases of disease. Several techniques were employed: immunohistochemical staining on muscle tissue and cultured skeletal muscle cells; flow cytometry, ELISA and chemiluminescence immunoassay on blood samples. Hypotheses regarding molecular mechanisms for muscle weakness and fatigue were tested in animal models, where we mainly used enzymatically dissociated muscle fibers and mechanically dissected muscle fibers to measure the force and calcium release from the sarcoplasmic reticulum under various defined molecular conditions.

**Results:** Based on previous observations on IL-1 expression in muscle tissue we first tested the role of IL-1 by using IL-1 blockade, anakinra, for 12 months in patients with refractory myositis. Eight of 15 patients had a clinical response which correlated to some response in biomarkers in blood, but muscle tissue inflammation persisted. Therefore, we searched for a new immune modulating target and found IL-15 to be expressed in muscle tissue of patients with myositis, and higher IL-15 expression was associated with more muscle dysfunction. Another approach was to test for the role of inflammatory molecules in early phases of muscle inflammation. In this context the extra nuclear presence of the alarmin high-mobility group box 1 (HMGB1) in the muscle fibers of patients with myositis without detectable inflammatory infiltrates is of interest. By *in vitro* experiments we showed that HMGB1 can influence muscle function by accelerating muscle fatigue and inducing MHC-class I expression via TLR4. Another receptor for HMGB1, TLR2, was also found in muscle tissue of patients with myositis and our animal study demonstrated that by knocking out TLR2 the skeletal muscle fibers became more muscle fatigue resistant.

**Conclusion:** Collectively, the investigations in my thesis suggest that inflammatory mediators play important roles in the pathogenesis of myositis and different molecules may contribute in different phases of disease. Hereby, HMGB1 might induce muscle dysfunction at an early stage of the disease via TLR4 (or/and TLR2). IL-15 could be involved in developing muscle dysfunction via maintaining T cell homeostasis in the muscles. IL-1 may be important in a subset of patients by driving the adaptive immune system. However, more studies are needed for a comprehensive understanding of their roles in order to develop new therapies.

## LIST OF SCIENTIFIC PAPERS

**I. Anakinra treatment in patients with refractory inflammatory myopathies and possible predictive response biomarkers.**

Mei Zong\*, Christina Dorph\*, Maryam Dastmalchi, Helene Alexanderson, Jennifer Pieper, Petra Amoudruz, Sevim Barbasso Helmers, Inger Nennesmo, Vivianne Malmström, Ingrid E. Lundberg. Ann Rheum Dis. 2014 May 1; 73(5):913-20.

**II. Effects of conventional immunosuppressive treatment on Interleukin-15 in muscle tissue of patients with polymyositis or dermatomyositis.**

Mei Zong, Ingela Loell, Eva Lindroos, Gustavo Nader, Helene Alexanderson, Christina Ståhl Hallengren, Kristian Borg, Snjolaug Arnardottir, Iain B McInnes and Ingrid Lundberg. Ann Rheum Dis. 2012 Jun; 71(6):1055-63.

**III. TLR4 as receptor for HMGB1 induced muscle dysfunction in myositis.**

Mei Zong, Joseph D. Bruton, Cecilia Grundtman, Huan Yang, Jian Hua Li, Helene Alexanderson, Karin Palmblad, Ulf Andersson, Helena E. Harris, Ingrid E. Lundberg and Håkan Westerblad. Ann Rheum Dis. 2013 Aug; 72(8):1390-9.

**IV. Loss of TLR2 increases fatigue resistance in mouse skeletal muscles**

Mei Zong, Arthur Cheng, Abram Katz, Niklas Ivarsson, Ingrid E. Lundberg, Håkan Westerblad, Helena E. Harris, Joseph D. Bruton. Submitted.

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## RELATED PUBLICATIONS

- I. Expression of HMGB1 and its receptors in hearts of patients with coronary artery disease with or without inflammatory rheumatic disease: A biopsy study.**  
Mei Zong, Ivana Hollan, Huiyuan Xiao, Cecilia Wick, Eva Lindroos, Knut Mikkelsen, Stein E. Rynning, Kjell Saatvedt, Sven M. Almdahl, Helena E. Harris, Ingrid E. Lundberg. Submitted.
- II. Pathogenesis, classification and treatment of inflammatory myopathies.**  
Mei Zong, Ingrid Lundberg. Nat Rev Rheumatol. 2011 May; 7(5):297-306. Epub 2011 Apr 5. Review.
- III. HMGB1 and Myositis.**  
Mei Zong, Helena E. Harris. Antioxidants & Redox Signaling. Review. Submitted.
- IV. Expanded T cell receptor V $\beta$ -restricted T cells from patients with sporadic inclusion body myositis are proinflammatory and cytotoxic CD28null T cells.**  
Jayesh Pandya, Andreas Fasth, Mei Zong, Snjolaug Arnardottir, Eva Lindroos, Vivianne Malmström and Ingrid E. Lundberg. Arthritis Rheum. 2010 Nov; 62 (11): 3457-66.
- V. Activated LTB<sub>4</sub> pathway in muscle tissue of patients with polymyositis or dermatomyositis.**  
Ingela Loell, Li Alemo Munters, Jayesh Pandya, Mei Zong, Helene Alexanderson, Andres Fasth, Christina Ståhl Hallengren, Olof Rådmark, Ingrid Lundberg, P-J Jakobsson and Marina Korotkova. Ann Rheum Dis. 2013 Feb; 72(2):293-9.

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# LIST OF ABBREVIATIONS

ALT = alanine transaminase  
AST = aspartate transaminase  
[Ca<sup>2+</sup>]<sub>i</sub> = free myoplasmic Ca<sup>2+</sup> concentration  
APC = antigen presenting cell  
Ca<sup>2+</sup> = calcium  
CD = cluster of differentiation  
CK = creatine kinase  
CNS = central nervous system  
COX = cytochrome c oxidase  
CXCL12 = C-X-C motif chemokine 12  
CXCR4 = chemokine C-X-C motif receptor type 4  
DC = dendritic cell  
DHPR = dihydropyridine receptor  
DM = dermatomyositis  
DMARD = disease-modifying anti-rheumatic drug  
DMEM = Dulbecco's Modified Eagle Medium  
ECG = electrocardiography  
EDL = extensor digitorum longus  
EMG = electromyography  
ER = endoplasmic reticulum  
ESR = erythrocyte sedimentation rate  
FBS = fetal bovine serum  
FDB = flexor digitorum brevis  
FI = functional index  
GC = glucocorticoid  
H&E = hematoxylin and eosin  
HAD = 3-hydroxyacyl-coenzyme A dehydrogenase  
HIF = hypoxia inducible factor  
HMGB1 = high-mobility group box 1  
IBM = inclusion body myositis  
ICAM = intracellular adhesion molecule  
IFN = interferon  
Ig = immunoglobulin  
IIMs = idiopathic inflammatory myopathies  
IL = interleukin  
IL-1R = interleukin-1 receptor  
ILD = interstitial lung disease  
i-IEL = intestinal intraepithelial lymphocytes

IS = immunosuppressive  
IVIG = intravenous immunoglobulin  
LDH = lactate dehydrogenase  
MAA = myositis-associated autoantibody  
MHC = major histocompatibility complex  
MMT = manual muscle test  
MRI = magnetic resonance imaging  
MSA = myositis specific autoantibody  
NFκB = nuclear factor kappa B  
NK = natural killer  
PBMC = peripheral blood mononuclear cell  
PBS = phosphate buffered saline  
PCr = phosphocreatine  
pDCs = plasmacytoid DCs  
PM = polymyositis  
RA = rheumatoid arthritis  
RAGE = receptor for advanced glycated end products  
rHMGB1 = recombinant HMGB1  
ROS = reactive oxygen species  
RyR = ryanodine receptor  
SLE = Systemic lupus erythematosus  
SR = sarcoplasmic reticulum  
TBS-T = Tris-buffered saline-Tween 20  
TCR = T cell receptor  
TLR = toll-like receptor  
TNF = tumor necrosis factor  
Treg = regulatory T cell  
VCAM = vascular cell adhesion molecule  
VEGF = vascular endothelium growth factor

## **1. Idiopathic inflammatory myopathies**

Idiopathic inflammatory myopathies (IIMs), briefly called myositis ("myo" means muscle and "itis" means inflammation), is the term for a group of rare, heterogeneous inflammatory diseases mainly affecting skeletal muscles. The IIMs are characterized clinically by muscle weakness and muscle fatigue, and histologically by the presence of inflammatory mononuclear cell infiltrates in muscle of the patients [1]. Glucocorticoids are the most commonly used medicine to treat patients with myositis, usually in combination with one or more immunosuppressive agents. Unfortunately, the beneficial effects are often limited and the adverse effects are prominent. These clinical observations indicate the need for development of new therapies. In recent years, a role of effector molecules/cytokines in the disease process has been suggested following the discovery of aberrant expression of some cytokines in muscle tissue of patients with myositis, such as tumor necrosis factor alpha (TNF $\alpha$ ) and interleukin (IL)-1. However, anti-TNF $\alpha$  treatment in patients with myositis has been tried but the reports to date show inconsistent results [2-5], and thus, further investigations about other cytokines and their potential role in disease mechanism are needed [6-11].

### **1.1. Classification**

Accurate classification of myositis is important in clinical studies to exclude involvement of misclassified patients with other myopathies and also sub-classification since different subgroups could have different response to conventional immunosuppressive treatment suggesting differences in underlying molecular mechanisms. Moreover, the clinical complications or prognosis could be different between subgroups. Currently, myositis is generally sub-classified into three main subgroups: polymyositis (PM), dermatomyositis (DM) and inclusion body myositis (IBM) based on patients' clinical and histopathological features [12-15], and this is also the classification that has been used in this thesis.

Since the 1980s, a variety of discoveries have begun to impact the sub-grouping of myositis; the myositis specific autoantibodies (MSA), further immunohistochemical characterization of muscle biopsies to include the presence of major histocompatibility complex (MHC)-class I expression in muscle fibers and subtyping of invading inflammatory cells [1, 16, 17]. Magnetic resonance imaging (MRI) has been used to visualize muscle inflammation and may also be helpful in the classification of myositis [18, 19]. To solve the unmet need for an updated and validated set of classification criteria, an international, multidisciplinary data driven project is ongoing within the frame-work of the International Myositis Assessment and Clinical Trials (IMACS) but the final results are not available yet.

### **1.2. Clinical manifestations and laboratory findings**

The incidence of myositis is around 5-7/100,000 per year. Both polymyositis and dermatomyositis are more often seen in females than in males (2:1) [20]. Muscle weakness and muscle fatigue are the most commonly reported clinical symptoms both in polymyositis and dermatomyositis. Their muscle problems usually develop slowly over months and mainly affect proximal muscle groups and are often symmetric. Patients typically report difficulties with daily activities such as climbing stairs, getting up from a bench which often requires the use of proximal muscles, while fine motor movements that depend on distal muscle groups, such as writing, drawing or buttoning a shirt are rarely affected, especially at the early stage of polymyositis and dermatomyositis. Muscle tenderness and myalgia could occur in some patients, usually in the early stage of the disease course in patients with polymyositis and dermatomyositis [1, 21]. Aside from these common clinical features, each subgroup of disease also has some typical clinical symptoms and signs that are described in detail in the following subsections.

### **1.2.1 Polymyositis**

Polymyositis usually has its onset at age 55-60 years old. It can occur alone or in association with other systemic autoimmune/connective tissue diseases in particular with systemic sclerosis and Sjögren's syndrome. The most frequent complaint of the patients with polymyositis is the weakness in the shoulder and pelvic girdle muscles. Weakness of neck muscles especially the neck flexor occurs in about half of the patients, while ocular and facial muscles are rarely affected. Dysphagia may develop secondary to esophageal muscle dysfunction or circopharyngeal obstruction. While myalgia and joint pain are usual, severe tenderness and synovitis are uncommon. Pulmonary and cardiac manifestations can occur at any time during the disease course [1, 21].

### **1.2.2 Dermatomyositis**

The clinical features described above for polymyositis can be found in patients with dermatomyositis. However, dermatomyositis is typically identified by characteristic skin rashes that can accompany, precede or follow the appearance of muscle weakness. The form of skin rash varies and can differ in different patients. It includes a heliotrope rash, blue-purple discoloration on the upper eyelids with edema, a flat red rash on the face and upper trunk, and erythema of the knuckles accompanied by a raised, violaceous scaly eruption (Gottron's sign and Gottron's papules). Differing from systemic lupus erythematosus (SLE) in which the phalanges are involved and the knuckles are spared, the erythema in dermatomyositis does not affect the phalanges. The erythematous rash can also appear on other parts of the body surfaces, including the knees, elbows, malleoli, neck and upper chest (V sign), or shoulders and back (shawl sign). The rashes can worsen after exposure to sunlight. The rashes may be very persistent but in some patients it also can be very faint and sometimes appear only transiently, in these cases, the diagnosis of dermatomyositis could be difficult. Dilated capillary loops at the base of fingernails are also

characteristic of dermatomyositis. The cuticles could be irregular, thickened, and distorted, and the lateral and palmar areas of the fingers may become rough and cracked with irregular, dirty horizontal lines (mechanic's hands). Mechanic's hands can also be seen in antisynthetase syndrome and is not specific for dermatomyositis.

### **1.2.3 Inclusion body myositis**

A more accurate term is sporadic inclusion body myositis (sIBM). sIBM is often misdiagnosed as polymyositis or some other myopathy disease, and is commonly identified when a patient previously diagnosed as polymyositis does not respond positively to conventional immunosuppressive treatment.

Sporadic inclusion body myositis is the most common acquired myopathy in patients above the age of 50 years old with the prevalence of 35/1,000,000 and a male-to-female ratio of 3:1 [22]. It differs from polymyositis and dermatomyositis in that muscle weakness and muscle fatigue usually involve both the proximal leg muscles and distal muscles, especially foot extensors and finger flexors. These manifestations are often found in the early phase of disease and are valuable clues in the clinical diagnosis of the patients. A further important difference from polymyositis and dermatomyositis is that muscle atrophy is more frequently and earlier observed in the patients with sIBM than patients with other forms of myositis. The muscle weakness and muscle atrophy can be asymmetric, with the selective involvement of the quadriceps, iliopsoas, triceps, and biceps muscles. Patients often complain of their susceptibility to fall due to collapse of their knees because of the quadriceps's weakness, or of difficulties in performing some fine motor movements, for example, holding a cup, turning a key or fastening a button owing to the weakness of their finger flexors. Head dropping is rarely observed. Sensory function such as touch is usually normal. The tendon reflexes could be normal especially at the early stages of the disease course, but can disappear in the late stages of the disease when the muscle atrophy becomes evident in the major muscle groups. The whole disease progression is usually quite slow and most patients with sIBM usually need some form of walking assistance after several years [7, 23].

Laboratory tests are commonly used to help the diagnosis and assessment of myositis, especially the laboratory evaluation of muscle-derived factors that reflect muscle injury and the determination of serum autoantibodies. Measurement of serum enzymes released from muscle has been used for many years and is still the most widely used tests for muscle injury. Elevated serum levels of creatine kinase (s-CK) are often found in myositis patients but normal s-CK levels can be shown in patients especially in advanced cases in which there is already significant muscle atrophy [24, 25]. Other enzymes, such as lactate dehydrogenase (LD), aspartate transaminase (AST) and alanine transaminase (ALT) could be also detected in elevated levels in the serum [25, 26].

Autoantibody, especially MSA testing is a useful tool in the diagnosis of myositis and more importantly some MSA are associated with a particular set of symptoms or organ involvements and can contribute to the treatment choice and the assessment of prognosis. The most frequent MSA is the anti-Jo-1 autoantibody, which is directed against histidyl-tRNA synthetase and is present in 20-25% of patients with polymyositis or dermatomyositis. Their levels correlate with disease activity and they are usually associated with a distinct clinical phenotype, antisynthetase syndrome [27, 28]. Altogether, eight types of antisynthetase autoantibodies have been found [28-32] and MSAs can also direct against other autoantigens. This series has been reviewed in our recent publication [11].

Muscle biopsy remains the gold standard for confirming the diagnosis of myositis [33]. The most typical changes in polymyositis are degeneration and regeneration of muscle fibers and CD8+ T cells invading non-necrotic fibers. In dermatomyositis, CD4+ T cells and B cells predominate in the perivascular areas and perifascicular atrophy [1, 21]. The typical features in sIBM are the “rimmed vacuoles” in the muscle fibers and Congo-red-positive amyloid deposits; otherwise it may appear identical to that of polymyositis. The “rimmed vacuoles” contain basophilic granular deposits and are easily demonstrated on cryostat sections stained with hematoxylin and eosin, while in paraffin sections they are difficult to observe owing to dissolution of the granular materials [1, 7]. However, despite the characteristic features described above, some patients may have a normal biopsy.

### 1.3. Diagnostic criteria

#### 1.3.1. Diagnostic criteria for polymyositis and dermatomyositis

Although there is currently no single universally accepted set of diagnostic criteria, there are some accepted principles as how to term or confirm the diagnosis of polymyositis and dermatomyositis. The most often used set was proposed by Bohan and Peter in 1975 [12]. The criteria they suggested are listed in Table 1.

**Table 1.** Diagnostic criteria for polymyositis and dermatomyositis by Bohan and Peter in 1975

<ol style="list-style-type: none"> <li>1. Symmetrical muscle weakness of limb-girdle and anterior neck flexors.</li> <li>2. Muscle biopsy revealing evidence of necrosis of type I and II fibers, phagocytosis, regeneration with basophilia, large vesicular sarcolemmal nuclei and prominent nucleoli, atrophy in a perifascicular distribution, variation in fiber size, and inflammatory exudates, often perivascular.</li> <li>3. Elevated serum levels of enzymes derived from skeletal muscle.</li> <li>4. Electromyography must show characteristics features.</li> <li>5. Characteristic cutaneous manifestation of dermatomyositis, including heliotrope rash or Gottron’s sign.</li> </ol>
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In addition the Bohan & Peter criteria require exclusion of several defined myopathies. The diagnosis of *definite polymyositis* is achieved when the first four criteria are met and there is no skin manifestation. The diagnosis of *probable polymyositis* is considered when three criteria are fulfilled without the rash. And a diagnosis of *possible polymyositis* is considered when two criteria are met without the skin rash.

Similarly, *definite dermatomyositis* can be used when three or four of these criteria are fulfilled including the skin rash. *Probable dermatomyositis* can be diagnosed with two criteria (plus the rash), and when one criterion plus the rash is found, *possible dermatomyositis* can be used.

### 1.3.2. Diagnostic criteria for sporadic inclusion body myositis

Different diagnostic criteria for sIBM have been suggested by different groups worldwide. The most frequently used were proposed by Griggs and colleagues in 1995 [14]. The main criteria are listed in [Table 2](#).

**Table 2.** Diagnostic criteria for inclusion body myositis by Griggs and colleagues (1995)

<ol style="list-style-type: none"> <li>1. Duration of disease <math>\geq 6</math> months.</li> <li>2. Age at the onset of the disease <math>\geq 30</math> years old.</li> <li>3. Muscle weakness must affect proximal and distal muscles of arms and legs and patient must exhibit at least one of the following features: <ol style="list-style-type: none"> <li>1). Finger flexor weakness.</li> <li>2). Wrist flexor weakness is more serious than wrist extensor weakness.</li> <li>3). Quadriceps muscle weakness.</li> </ol> </li> <li>4. Serum creatine kinase <math>&lt; 12</math> times normal</li> <li>5. Muscle biopsy with the following features: <ol style="list-style-type: none"> <li>1). Inflammatory myopathy characterized by mononuclear cell invasion of non-necrotic muscle fibers.</li> <li>2). Vacuolated muscle fibers.</li> <li>3). Intracellular amyloid deposits or tubulofilaments by electron microscopy.</li> </ol> </li> <li>6. Electromyography must display consistent features of inflammatory myopathies.</li> </ol>
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*Definite inclusion body myositis* can be diagnosed with the typical findings of muscle biopsy irrespective of other clinical features. In contrast, *possible inclusion body myositis* can be diagnosed by the presence of characteristic clinical and laboratory features but in the absence of the typical histological findings in the muscle biopsies from an individual.

### 1.4. Treatment

Due to the lack of a thorough understanding of disease mechanisms, there is no specific therapy for myositis patients so far. The main aims of the treatment are mainly to relieve

the symptoms, improve muscle function, slow disease progress, reduce or prevent more organ damage and provide necessary support so the patients can keep a relatively normal life as long as possible.

Traditional pharmacological therapies are based on immunosuppression but are largely non-specific and run the risk of generalized immunosuppression. There are only a few controlled trials of treatment in polymyositis and dermatomyositis patients and therefore treatment recommendations are mainly based on clinical experience and open trials as summarized previously [8]. Introduction of glucocorticoids in the 1950s had a major impact with reduced morbidity and mortality and they are still the fundamental basis of treatment. High doses of glucocorticoids, 0.75-1 mg/kg/day, are often given for several weeks. Many patients respond with improved function, but few recover their former physical performance, and unwanted side effects are common. Therefore, many experts recommend a combination of glucocorticoids with another immunosuppressive drug to get a better response. The most commonly used first line agents are methotrexate 15-25 mg/week or azathioprine 2 mg/kg/day. If these are not tolerated or are without effect the next most recommended second line agents are: cyclosporine A which was found to be equally effective as methotrexate [34], or mycophenylate mofetile, as supported by case reports[35-37]. One of the few placebo controlled trials that showed clear beneficial effects in treatment resistant dermatomyositis combined high dose intravenous immunoglobulin with conventional immunosuppressive agents for three months [38]. However, a more recent open study including treatment resistant DM, PM and IBM patients could not confirm this effect in any of the three myositis subsets; neither on clinical out-come measures nor on the inflammatory changes in muscle tissue [39]. In IBM the effects of pharmacological treatment were limited and to date there is few data as to the beneficial effects of immunosuppressive treatment in IBM. Thus, there is a clear need for new therapies for all forms of myositis and such development depends on improved knowledge on the key molecular pathways in these diseases.

In recent years beneficial effects of increased physical exercise in addition to conventional immunosuppressive treatment have been demonstrated [40-42]. In a pilot study, a modest amount of resistant exercise led to improved clinical performance and in muscle tissue there was a reduced expression of genes involved in inflammation and fibrosis [43]. These findings indicate that exercise in established polymyositis or dermatomyositis may have a beneficial effect on molecular expression in the target organ of myositis. This needs to be replicated in larger studies.

The new biological agents permit targeted therapy, and thereby they will be helpful in understanding key molecular pathways in diseases, as has been the case in the usage of



TNF blockade to treat rheumatoid arthritis (RA). However, the experience of treatment with targeting biological agents in myositis is still limited.

## 2. Disease mechanisms

Immune mediated mechanisms are regarded to play important roles in the pathogenesis of myositis, which was supported by the observations of abundant expression of immune cells and effector molecules/cytokines in muscle tissue of patients with myositis [11]. T cells and macrophages are two major groups of immune cells that have been reported in muscle tissue of all subgroups of patients with myositis [44, 45]. Due to the prominent finding of both CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells a role of T cells in the disease mechanisms of myositis has been proposed [46-48]. T cells may have direct myotoxic effects targeting muscle fibers and leading to necrosis. Both T cells and macrophages may have indirect effect on muscle fibers through production of molecules such as cytokines which may affect the muscle fiber phenotype including the characteristic MHC-class I expression in muscle fibers of patients with myositis. In addition, both endothelial cells and muscle fibers may contribute to a proinflammatory environment by production of cytokines in muscle tissue of patients with myositis. This is particularly interesting in the context of cases where no inflammatory cells can be detected in a muscle biopsy. From clinical practice this is a well-known observation and can partly be explained by sampling error, but, in addition, there is no correlation between the degree of inflammatory infiltrates and muscle fiber necrosis and the degree of muscle weakness suggesting that other mechanisms than direct T cell mediated myotoxicity may contribute to muscle weakness and this could be by effects on muscle fiber contractility from molecules in the inflammatory environment in the muscle as supported from previous observations that TNF may affect muscle fiber contractility inducing muscle weakness [49]. Another interesting observation from longitudinal studies is the persistence of T cells in muscle tissue in patients with persisting muscle weakness despite conventional immunosuppressive treatment given for several months. This observation has been addressed in our research group and a striking predominance of a subphenotype of T cells of both CD4<sup>+</sup> and CD8<sup>+</sup> lineage of T cells, so called CD28<sup>null</sup> T cells have been observed in muscle tissue and peripheral blood in patients with PM, DM and IBM. These cells have restricted T cell receptor patterns suggesting that they are antigen driven and are apoptosis resistant which may contribute to their persistence in muscle tissue despite treatment. All together previous studies suggest that both T cell mediated muscle weakness as well as other mechanisms may contribute to the clinical symptoms in these patients.

TNF was the first cytokine to be targeted by treatment in patients with myositis due to the success of TNF-blockade in rheumatoid arthritis. However, the effects have been contradictory [2-5]. In one open study using infliximab treatment, some patients even flared and induction of type I interferon activity was recorded arguing against TNF as a key molecule in chronic resistant myositis [2]. Therefore the importance of TNF is still controversial in myositis.

Overexpression of IL-1 $\alpha$ , IL-1 $\beta$  and their receptors was also demonstrated in muscle tissue from most patients with PM, DM and sIBM [50, 51]. Moreover, some beneficial effects of IL-1 blockade were reported in a case study of one myositis patient with anti-Jo-1 autoantibodies [52]. These data suggest a role for IL-1 in the pathogenesis of myositis. Based on these observations we initiated an open label-mechanistic study with anakinra treatment in fifteen patients with refractory myositis (**Paper I**). The detailed results in this study are presented in the “Result and discussion” part of this thesis. One interesting finding was the persisting inflammation including T cells homeostasis in muscle tissue after treatment, even in the patients who had a good clinical response to anakinra. This persistence of T cells is consistent with our early observation that while macrophages decreased or even disappeared after glucocorticoids treatment, T cells in muscle tissue were usually maintained [53, 54]. As the most important T cell-growth factor, IL-2 is rarely found in muscle tissue of patients with myositis, it is possible that other cytokine (s) may have IL-2-like functions stimulating T cell proliferation and differentiation. In this context we focused our interest on IL-15 (**Paper II**) as it shares some similar functions with IL-2 [55-59].

To address the question on other molecular mechanisms that may have a role in causing muscle weakness than those that can be explained by T cells our research group had previously found that the alarmin high mobility group box protein (HMGB) 1 which is constitutively expressed in all nucleated cells was abnormally expressed in the cytosol of muscle fibers in patients with myositis. Moreover, based on animal studies it was demonstrated that HMGB1 can induce MHC-class I expression and accelerate muscle fatigue by reducing calcium release from sarcoplasmic reticulum [9, 10]. Therefore HMGB1 may be a molecule that can affect muscle fiber function and this could be independent of presence of T cells. However, the mechanisms how HMGB1 could possibly act on muscle fibers were not clear and needed to be investigated (**Paper III**). Toll-like receptors (TLRs) including TLR2, TLR4 and receptor for advanced glycosylated end products (RAGE) are known receptors for HMGB1 signaling. During our exploration about how HMGB1 mediates muscle dysfunction in paper III, we found some interesting muscle function modifications after the TLRs were knocked out, in which TLR2 knock-out mice raised our further interest that we wanted to explore to get a better understanding of muscle fiber fatigue (**Paper IV**).

In the following parts the four inflammatory molecules (IL-1, IL-15, HMGB1 and TLR2) that were investigated in this thesis are introduced in more detail regarding their basic properties and potential roles in myositis.

## 2.1. IL-1

The IL-1 gene family includes three members: IL-1 $\alpha$ , IL-1 $\beta$ , and IL-1 receptor antagonist (IL-1Ra) and two IL-1 receptors: IL-1RI and IL-1RII. The binding of IL-1 $\alpha$  or IL-1 $\beta$  with

either receptor will initiate a signal transduction while IL-1Ra acts as a competitive inhibitor [60-62]. IL-1 is mainly produced by activated macrophages and by endothelial cells and may increase the expression of some pro-inflammatory genes [51, 63, 64]. One role relevant to disease is its ability to increase expression of adhesion molecules such as intracellular adhesion molecule (ICAM)-1 on mesenchymal cells and vascular cell adhesion molecule (VCAM)-1 on endothelial cells, both of which have been found to be increased in muscle tissue in patients with myositis [65-68]. IL-1 $\alpha$  can also suppress myoblast proliferation and myoblast fusion, leading to poor muscle cell regeneration [69]. IL-1 receptors are expressed on endothelial cells [70] and more recently were also demonstrated to be expressed and co-localized with its reciprocal ligands, IL-1 $\alpha$ , IL-1 $\beta$  and IL-1Ra on muscle fiber membranes in PM and DM patients, lending further support for a role of IL-1 in the pathogenesis of myositis [50].

IL-1 $\alpha$  and IL-1 $\beta$  have been found to be consistently expressed in muscle tissue of PM, DM and IBM patients [50, 51, 63, 64, 71-76]. Elevated levels of these cytokines have been found in muscle tissue of patients with impaired muscle performance, both in the early phase before treatment and in a chronic phase of disease with persisting muscle weakness, indicating that IL-1 could be a potential target of therapy in myositis [51, 63, 64]. Further support for a role of IL-1 in myositis are the elevated serum levels of IL-1Ra mRNA and protein in patients with active PM and DM compared with inactive PM and DM and normal controls [77]. Serum IL-1Ra levels have also been shown to be significantly higher in PM and DM compared to patients with spondylarthropathies and normal controls. IL-1Ra levels were particularly elevated in patients with active myositis and decreased in response to therapy [78]. Moreover, the IL-1Ra A1 allele in Caucasians and the A3 allele in African-Americans was found to be a risk factor for juvenile myositis compared to normal race-matched controls [79].

Anakinra, a recombinant IL-1Ra, was recognized as the most effective current therapy for hereditary autoinflammatory syndromes related to mutations in the inflammasome component cryopyrin/NALP3 [80-82]. It has also shown promising results in the treatment of systemic-onset Still's disease [83, 84]. In case of myositis there is very limited information. A case-report showed a rapid reduction of fever, polyarthritis and acute phase reactants following administration of anakinra in a patient with antisynthetase syndrome [85]. However, in a second case report on lupus myositis, there was no obvious effect of anakinra treatment [86]. Until recently there were no reports based on a larger group of patients with myositis. Therefore, we investigated the effects of anakinra in myositis patients in a pilot study of fifteen patients with refractory PM, DM and IBM.

## **2.2. IL-15**

IL-15 belongs to the 4 $\alpha$ -helix bundle cytokine family with some structural homology to IL-

2. IL-15 mediates its functions via its receptor that consists of three components, IL-2R $\beta$ , common gamma chain ( $\gamma$ c) and IL-15R $\alpha$ . IL-15R $\alpha$  has a very high affinity for IL-15 [55-59]. Traditionally it has been thought that IL-15 functions like other classic interleukin cytokines, such as IL-1, which can bind to its receptors and initiate downstream functions. But recent studies show that the way that IL-15 acts is quite different from others. It goes by the way called trans-presentation, which means that IL-15R $\alpha$ -producing cells bind IL-15 and then present it to cells expressing IL-2R $\beta$  and  $\gamma$ c subunits [57, 87, 88].

Originally IL-15 was described as a T-cell-growth factor that promotes the proliferation of T cells in a fashion akin to that of exogenous IL-2 [58]. Stimulation of T cell receptor (TCR) with IL-15 has been shown to induce various activation antigens, such as CD69 [89]. IL-15 was shown to protect concanavalin A-activated human T lymphoblasts from undergoing apoptosis after Fas or CD3 cross-linking or treatment with dexamethasone *in vitro* and *in vivo* [90]. Later additional roles of IL-15 during development of a variety of cells were also revealed, such as natural killer (NK) cells, NKT cells, and intestinal intraepithelial lymphocytes [91].

IL-15 knock-out mice showed a lack of NK cells, deficiency in NKT cells (thymic and peripheral), deficiency in memory CD8<sup>+</sup> T cells, decreased intestinal intraepithelial lymphocytes (i-IEL) TCR $\gamma\delta$  cells and i-IEL CD8 $\alpha$  cells [92]. IL-15R $\alpha$  knock-out mice showed absence of NK cells, deficiency of NKT cells (30% of wildtype (WT)), deficiency of single-positive CD8<sup>+</sup> T cells (thymus), deficiency of CD8<sup>+</sup> T cells (periphery), and deficiency of memory CD8<sup>+</sup> T cells [93].

Both IL-15 and IL-15R $\alpha$  have a widespread expression at the transcriptional level in a variety of normal human tissues and cells including skeletal muscle [57]. But due to strict posttranscriptional regulations, IL-15 and IL-15R $\alpha$  are rarely detected at the protein level in normal tissues or organs [88]. However, several groups have reported the expression of IL-15 in various tissues of patients with infectious diseases, cancer, autoimmune or inflammatory disorders such as RA where IL-15 protein was demonstrated in synovial fluids and synovial membranes [87, 94]. Agostini et al suggested a potential role for IL-15 in the pathogenesis of pulmonary sarcoidosis as alveolar macrophages isolated from patients with active sarcoidosis expressed IL-15 mRNA and cytoplasmic or membrane IL-15 protein, whereas macrophages isolated from patients with inactive disease or normal donors did not. In addition, CD4<sup>+</sup> T cells isolated from bronchoalveolar lavage of patients with active sarcoidosis expressed components of the IL-2/15R complexes and proliferated in response to IL-15, suggesting that macrophages may provide a proliferative signal to T cells in the lungs during this disease process [95]. Patients with multiple sclerosis were found to have higher numbers of IL-15 mRNA-expressing blood mononuclear cells compared with patients with aseptic meningoencephalitis or healthy controls. Increased

numbers of IL-15 mRNA-expressing cells were found in the central nervous system compared to the blood of multiple sclerosis patients [96]. In addition, patients with chronic, progressive multiple sclerosis had higher IL-15 expression than those with relapsing, remitting multiple sclerosis. Later study confirmed this finding and also correlated it with the duration and durability of multiple sclerosis [97].

A potential role of IL-15 in myositis is firstly highlighted by the finding of overexpression of IL-15 at the protein level in the muscle tissue of patients with PM and DM. One report based on three PM and two DM patients showed that IL-15 was predominantly expressed in muscle fibers and IL-15 was also expressed in myoblasts derived from muscle biopsies from these patients [98, 99]. High serum levels of IL-15 in PM and DM patients have also been reported [100]. All these observations suggest a potential role of IL-15 in PM and DM. Furthermore, as we mentioned earlier, T cells in muscle tissue of patients with myositis keep homeostasis even after glucocorticoid or anakinra treatment [53], while the most important T cell-growth factor, IL-2, is absent. Therefore, it is interesting to detect whether IL-15 can promote the proliferation of T cells in a fashion akin to that of exogenous IL-2 in myositis.

### **2.3. HMGB1**

HMGB 1 is a ubiquitous non-histone protein present in the nucleus of cells. Inside the nucleus HMGB1 binds DNA and helps to regulate transcription and chromatin structure. However, when the cells are activated or die, HMGB1 can translocate from the nucleus to the cytosol and may be released out of the cell. Extracellular HMGB1 has been described to mediate different functions including chemotaxis, cytokine induction, and tissue damage or healing [101-103]. HMGB1 does not act via one unique receptor but rather uses receptors that are known to also bind other ligands. To date the most investigated are RAGE, TLR2 and TLR4 [104, 105]. Recent studies also show that not only its cellular or extracellular location but also the redox statuses of three critical cysteines at positions 23, 45 and 106 determine the biological activities of HMGB1. When all three cysteines are fully reduced, HMGB1 mediates chemotactic activity by forming a heterocomplex with CXCL12 and signals via CXCR4 in a synergistic fashion compared to CXCL12 alone. However, this HMGB1 isoform does not activate cells to produce cytokines. Disulfide HMGB1 (a disulfide bond formed between C23 and C45) will induce proinflammatory cytokine release via the TLR4 receptor signaling pathway. However, further oxidation of any of the cysteine residues will eliminate all proinflammatory activities [106, 107]. In addition, HMGB1 can promote inflammation by forming immunostimulatory complexes with other molecules including IL-1, endotoxin and DNA and additional molecules [108-113]. HMGB1 with its partner molecules can act synergistically via the receptors of the partner molecule. The mechanism underlying this synergy is presently not well understood.

HMGB1 can drive the pathogenesis of infectious diseases as well as autoimmune and auto-inflammatory diseases [101-103, 114-116]. Elevated levels of total HMGB1 have been demonstrated in the serum, synovial tissue and synovial fluid of patients with RA [117, 118]. Blocking HMGB1 signaling can attenuate inflammation in experimental arthritis models [119-121]. In SLE, systemic HMGB1 levels are increased and correlate well with disease activity [122-125] and treatment based on HMGB1 antagonists ameliorates the course and outcome in experimental lupus [126]. Recently, studies of HMGB1 in myositis have also been reported. Cytoplasmic HMGB1 expression was demonstrated in inflammatory cells, endothelial cells and even muscle fibers in muscle tissue of patients with myositis, suggesting a possible role of HMGB1 in the pathogenesis of myositis [9, 10].

## **2.4. TLR2 and TLR4**

TLR2 and TLR4, two members of the toll-like receptor family, are traditionally considered to function as membrane proteins and expressed abundantly on the surface of certain peripheral blood leukocytes [127, 128]. They are well described as playing important roles in the recognition of pathogen-associated molecular patterns (PAMPs), such as lipopeptides and lipopolysaccharide from bacteria [129]. Activation of TLR2/4 by PAMPs initiates innate immune responses, triggers intracellular signaling pathways via myeloid differentiation primary response protein 88 (MyD88) and leads to the production of cytokines and chemokines. They can be considered to represent the first line of host defense against pathogens and plays an important role in both innate and adaptive immunity [130, 131].

Recently several new discoveries have added to our existing knowledge about these TLRs. First, in addition to their expression on the immune cells, they have also been found in non-immune cells including skeletal muscles where TLR2 and TLR4 were detected at both the RNA level with polymerase chain reaction (PCR) and the protein level via western blot [132, 133]. Second, TLR2 and TLR4 recognize not only PAMPs but also damage-associated molecular patterns (DAMPs), one of which is HMGB1 [129]. As mentioned above, HMGB1 is a very conserved and ubiquitous protein normally found in nuclei of cells. HMGB1 can be released from nuclei and from cells under certain conditions and this extranuclear/extracellular HMGB1 can induce tissue damage and inflammation via binding to its receptors including TLR2 and TLR4 [134-136].

Based on these advances in our knowledge we postulated that TLR2/4 signaling not only exerts important functions in fighting infections but could also play important roles in non-infectious disorders, such as autoimmune disease where they could bind endogenous ligands such as HMGB1, triggering inflammation and initiating tissue damage. A recent report showed that TLR2 expression on skeletal muscles is correlated with insulin

resistance and inhibition of TLR2 improves insulin sensitivity [133], suggesting that TLRs can play multiple roles in skeletal muscle pathology.



### 3. Aims of this thesis

The overall aim of this thesis was to achieve an increased knowledge of the molecular mechanisms in myositis that can help to develop new target therapies in myositis in the future. This thesis is based on four studies which describe potential roles of four different inflammatory molecules in the disease development of myositis.

The specific aims were:

- **Paper I:** To investigate if IL-1 has a role in the disease mechanisms by targeting IL-1 by anakinra, an IL-1 receptor antagonist, in a mechanistic study and to identify possible predictive biomarkers of this treatment.
- **Paper II:** To investigate the potential role of IL-15 in the disease mechanisms of myositis by investigating whether IL-15 and its specific receptor IL-15R $\alpha$  are expressed in muscle tissue of patients with myositis, the effects of immunosuppressive treatment on their expression, and furthermore, the relation of IL-15 to the clinical symptoms.
- **Paper III:** To investigate the potential role of HMGB1 in the disease mechanisms, in particular on muscle fiber function, that is how HMGB1, an alarmin that is abnormally expressed in muscle tissue of patients with myositis, may mediate muscle dysfunction.
- **Paper IV:** To investigate a potential new mechanism for muscle fiber dysfunction that is through TLR2, a known defense molecule of the innate immune system by investigating its effect on muscle fiber contractility in an animal model and by investigating its expression in patients with myositis in relation to clinical symptoms.

## **4. Patients**

All the patients who participated in our studies were recruited from the rheumatology unit at Karolinska University Hospital, Sweden. All studies were approved by the Regional Ethical Review Board in Stockholm and all patients and control individuals gave their written informed consent to participate in the study. Patients are being subject to standardized follow up including assessment of disease activity and muscle performance.

### **4.1. Patients in Paper I**

Fifteen patients (8 females and 7 males; median age=58 years, range 45-77; median disease duration=7 years, range 10 months-19 years) with refractory myositis were included in the study. All patients had previously been treated with high-dose prednisolone in combination with azathioprine (AZA) or methotrexate (MTX). Six patients had definitive PM and four had definitive DM according to Bohan and Peter's criteria [12, 13] five patients had IBM according to Grigg's criteria [14].

All patients were given self-administered anakinra subcutaneously with a daily dosage of 100 mg for 12 months. Allowed concomitant treatment included glucocorticoids, AZA, MTX or anti-malarial drugs in stable doses during the trial. A positive response was defined according to the suggestion of IMACS as  $\geq 20\%$  improvement in three or more of the six core set parameters of disease activity and no more than two worsening  $\geq 25\%$  which could not include manual muscle test (MMT)-8. Worsening was defined by  $\geq 30\%$  reduction in any three of the core set parameters. Improvement of functional index (FI) was defined as  $\geq 20\%$  increase in FI whereas worsening was defined as  $\geq 20\%$  reduction in FI [79, 137-139].

We investigated blood samples by ELISA and muscle biopsies by immunohistochemistry from these patients before and after treatment in order to find out some possible predictive response markers and to get information on whether IL-1 could have a role in the pathogenesis of myositis.

### **4.2. Patients in Paper II**

A cohort of seventeen patients (12 women and 5 men; median age 60 years, range 41-88) with PM (n=9) or DM (n=8) according to the Bohan and Peter criteria were included in this study [12, 13]. All patients were recently diagnosed and muscle biopsies were taken at the first assessment for the study. They were treated with high initial doses of glucocorticoids, slowly tapered over the first year. Additional agents were administered according to the treating physician's decision which included MTX, AZA, or cyclophosphamide. The second biopsy was obtained after average 8 months' immunosuppressive treatment (range 4-16 months). Clinical outcome was measured by FI, MMT-8 and serum creatine

phosphokinase (s-CK). Seven healthy individuals were also included in this study as controls (5 women and 2 men; median age 57 years, range 26-78, one patient's age information is missed).

Muscle biopsies from these seventeen patients before and after 8 months' conventional immunosuppressive treatment and seven healthy individuals were investigated by immunohistochemistry using antibodies against IL-15 and IL-15R $\alpha$ . Quantification was performed by computerized image analysis. Cellular localization of IL-15 was determined by double immunofluorescence.

#### **4.3. Patients in Paper III**

Fourteen patients (8 females and 6 males, median age 46 years, range 26-61) with short duration of muscle weakness (median 4 months, range 1-9) were included in this study. Muscle biopsies used in this study were selected from patients in the early stages of disease, where there were no obvious signs of cellular infiltrates in muscle tissue. The myositis diagnosis was based upon cumulative clinical and laboratory findings according to Bohan and Peter criteria [12, 13]. A later muscle biopsy confirmed the diagnosis (PM=8; DM=6) by presence of inflammation and/or signs of muscle fibre damage. Clinical outcome was measured by the FI and serum s-CK. Five healthy individuals (4 females and 1 male, median 63 years, range 50-78) without any overt clinical or histopathological signs of muscle disease were also included in our study as control.

Muscle biopsies from the patients and healthy individuals were investigated by immunohistochemistry using antibodies against TLR4 and MHC-class I. Quantification was performed by computerized image analysis.

#### **4.4. Patients in Paper IV**

Six patients diagnosed with polymyositis or dermatomyositis [12, 13] with established disease and persisting muscle weakness despite conventional immunosuppressive treatment (5 women and 1 men; median age = 69.5 years, range 55-75; median disease duration=47 months, range 18 months-102 months) were included in this study. They were selected for having few inflammatory cell infiltrates in their muscle biopsies. Muscle biopsy samples from six healthy, age-matched controls (4 women and 2 men, median age 68.5 years, range 42-77) without clinical or histopathological signs of any muscle disease were included as controls.

Muscle biopsies from these patients and healthy individual controls were investigated by immunohistochemistry using antibodies against TLR2.

## **5. Methods**

### **5.1. Measurement of muscle dysfunction in patients with myositis**

Weakness and fatigue are terms that are often used as if they mean the same thing, but in fact they describe two different perceptions. It is important to know exactly what is meant when the patients say “feel weak” or “feel fatigued” because it can help the doctor narrow down the possible causes of the symptoms.

Muscle weakness is a lack of physical or muscle strength and the feeling that extra effort is required to move your arms, legs, or other muscles. If muscle weakness is the result of pain, the person may be reluctant to use the muscles as it will hurt. General weakness often occurs after you have done too much activity, such as by taking an extra-long cycling or hiking trip. MMT is regarded as a reflection of the muscle strength of patients. One suggested version of MMT to be used in myositis is the MMT that measures strength in eight muscle groups (MMT-8) with a maximum score of 80. Detailed information was described in a previous publication [140].

Muscle fatigue is defined as the point when a muscle fails to maintain the required or expected force to perform a given activity. Thus, fatigue is not the total exhaustion or a complete inability to exert force, but rather more a subtle phenomenon where impaired muscle function is demonstrated. Although the presence or development of fatigue is a hindrance to physical activity, it may serve as a protective function against muscle damage. Myositis functional index (FI) is regarded as a fine measurement for muscle fatigue. It measures the number of repetitions performed in defined muscle groups on both sides of the body. The individual's total score is presented as the percentage of the maximal score 64 [141] .

### **5.2. Skeletal muscle biopsies from myositis patients**

Muscle biopsy is a very important tool for diagnosis of myopathies. Identification of histopathological changes in the muscle tissues obtained from the patients plays an essential role in the diagnosis when myositis is suspected, and helps to exclude other myopathies. Therefore, a simple and easily used muscle biopsy technique is important, one such being the percutaneous conchotome muscle biopsy technique [142] that has been used in Sweden both for diagnostic and research purposes. This technique gives a good-sized muscle tissue sample that allows for diagnostic evaluation in myositis patients and is also sufficient for additional research purposes, such as immunohistochemistry, western blot or gene expression detection. It is a simple procedure, easy to perform by the clinician. For the patients it is also safe with a low complication rate and minimum discomfort. The method serves as a diagnostic tool and by performing repeated biopsies one can assess the effect of a given therapy on molecular expression in muscle tissue and correlate this finding with clinical outcome measures.

### **5.3. Skeletal muscle sample cryopreservation and cryosection**

Proper freezing and storing of muscle biopsies is very important as it has been found that lots of damage and freezing artifacts can be produced when the muscle samples are not frozen properly. Reliable immunohistochemistry staining becomes very difficult because lots of unspecific staining can be found and the muscle morphology is not good enough for evaluation. To avoid these problems and to have good muscle samples for immunohistochemistry study, we used the following method to freeze and store our samples and to prepare the sections for the staining.

Once we get the muscle tissues, they are put on a physiologically buffer (0.9% saline) presoaked cloth for 15 minutes sitting on watery ice. Then the muscle tissue was put into isopentane which has been prechilled by liquid nitrogen. Notice directly placing the muscles into liquid nitrogen disrupts muscle morphology because of non-uniform freezing and should be avoided if you want to perform immunohistochemistry staining. After about one minute in the isopentane, the samples were moved directly into a tube on dry ice (around -70 °C). Before putting these samples into -80 °C freezer for long-term storage, some small pieces of watery ice were added to the tubes in order to prevent the samples from freeze drying in the future. Repeated freezing and thawing of samples should be avoided as it can damage the morphology.

When making cryostat sections from the biopsies, the tissues were kept on dry ice. The tissues were mounted on OCT medium and then sectioned. After the sections were obtained, they were dried at room temperature for 30 min. The slides were then fixed according to characteristics of the antigens to be investigated. Generally, for cell surface antigens, acetone fixation was used. Slides were placed in 50% cold acetone for 30 seconds, and then 3 minutes in 100% cold acetone. For intracellular antigen detection, slides were placed in 2% formaldehyde +4 °C in darkness, washed twice in cold PBS, and then the slides dried at room temperature and were stored at -80 °C or were used for staining.

### **5.4. Immunohistochemistry and immunofluorescence**

Immunohistochemical staining is a good technique to demonstrate molecules on muscle tissues at protein level. In our lab, by usage of formaldehyde or acetone fixation we can detect intracellular or cell surface markers respectively. We use saponin as a permeabilizing detergent when detecting intracellular markers. 1% H<sub>2</sub>O<sub>2</sub> blockade is always performed before application of primary antibody. 1% normal serum from the same species as the secondary antibody comes from is recommended before or after primary antibody application. Routinely, after the secondary antibody incubation, avidin-biotin-peroxidase complex (ABC elite, PK-6100, Vector) is applied for 45 minutes and then reactions are developed using a peroxidase substrate kit (SK-4100, Vector) containing 3, 3'-

diaminobenzidine (DAB). We use Mayer's hematoxylin counterstaining to show the nuclei of the cells. Slides were mounted using glycerol at the end for observation under a microscope.

To obtain a nice and reliable staining primary antibody should be carefully chosen and they should be titrated on recommended positive tissues/cells in order to know the good concentration and suitable protocol for the staining in muscle tissue. If the background is a problem, adding a step of avidin/biotin blockade or enhancing the serum concentration in the serum blockade step could be considered. Including a negative control with isotype staining and a positive control is necessary and helpful.

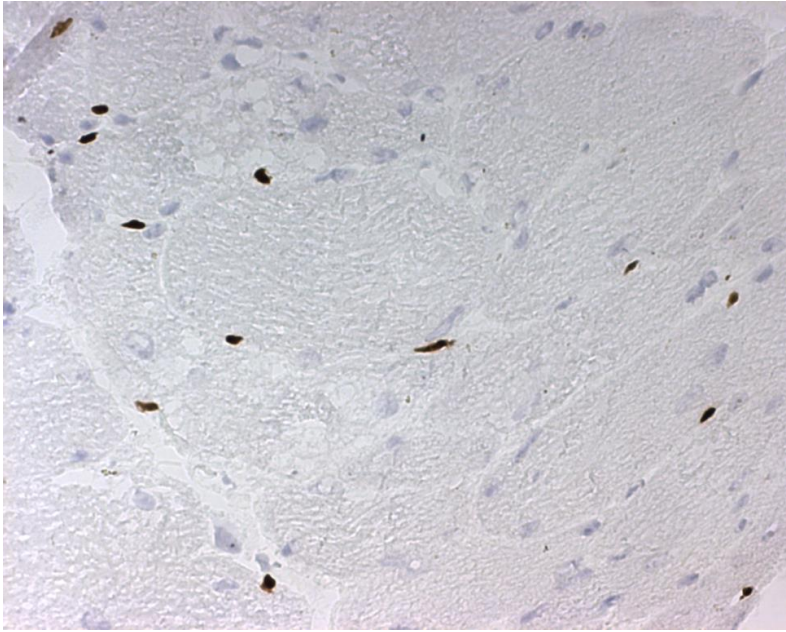
Immunofluorescence is used in some of our studies in order to distinguish staining to different cellular structures. The staining procedure is similar with immunohistochemistry. The important issue is that the two primary antibodies should come from different species. And all the steps should be performed in the dark as much as possible after fluorophore application.

In our lab, stained tissue sections were examined using a Polyvar II microscope and a digital Leica camera 300F. The expression of positive markers was quantified on the whole tissue sections by computer-assisted analysis with a Quantimet 600 image analyzer usually by two independent observers who were blinded to the identity of specimens. The average scores from the two assessments were used for statistical analysis. Positive staining was either calculated as the number of positive cells per  $\text{mm}^2$  tissue area or as the percentage positively stained area of total counterstained tissue area ( $\text{mm}^2$ ).

### **5.5. Skeletal muscle cell culture**

In order to develop skeletal muscle cell culture, it is necessary to introduce myosatellite cells. Myosatellite cells are adjacent to the sarcolemma of a differentiated muscle fiber and situated between the basement membrane (endomysium) and sarcolemma (cell membrane) of individual muscle fibers. Satellite cells are able to differentiate and fuse and augment existing muscle fibers and also to form new fibers. They are involved in the normal growth of muscle and in muscle regeneration following injury or disease. In undamaged muscle tissue, the majority of myosatellite cells are quiescent which means they neither differentiate nor undergo cell division [78, 143, 144]. Myosatellite cells express distinctive genetic markers. It is known that all myosatellite cells express Pax7 and Pax3. Activated satellite cells are known to express myogenic transcription factors, such as Myf5 and MyoD. They also express muscle specific filament proteins such as desmin [145-147].

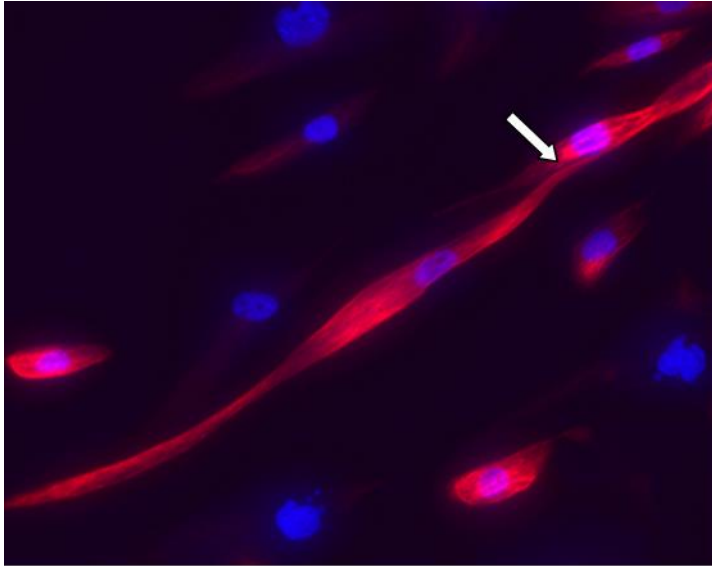
Figure 1 shows the Pax7 staining of satellite cells in the muscle tissue of a patient with myositis.



**Figure 1.** Pax7 staining of satellite cells in the muscle tissue of a patient with myositis. Brown color shows Pax7 positive satellite cells. Blue color demonstrates nuclei of muscle fibers and infiltrating cells. Magnification 20x.

It is extremely complicated to perform interventions on human skeletal muscle fibers *in vivo*. *In vitro* experiments on human cultured muscle cells can be used to address some research questions. These muscle cells can be differentiated from satellite cells in muscle tissue from patients or healthy individuals. Then investigations can be done *in vitro* based on these cultured cells. Although it cannot completely represent the situation *in vivo* but so far it is the best way to examine mechanisms at present.

The muscle cell culture procedure can be divided into two different stages based on the stage of muscle development and are referred to myoblast and myotubes. Myoblast is a muscle precursor exhibiting reasonably similar metabolic characteristics to adult skeletal muscle; but it does not physically resemble a differentiated muscle fiber or myotube. Myoblasts can differentiate into myotube (Figure 2), usually by starving them by reducing serum in the culture medium. The distinct morphological difference between myoblasts and myotube is that myoblast is mononuclear cell while myotube is multinucleated. As myoblasts fuse, they undergo a dramatic switch of phenotype that depends on the coordinated activation of a large number of muscle-specific genes and increased expression of skeletal muscle specific proteins such as myosin and MyoD. In addition to cultivate and differentiate muscle cells from human muscle tissue there are several muscle cell lines available on the market; the most often used are C2C12 which is from mouse skeletal muscle, and L6 derived from rat skeletal muscle.



**Figure 2.** An immunofluorescence staining picture showing the myotube forming process where two myoblasts are trying to fuse with each other (arrow). Desmin demonstrate the myoblasts (red color), blue color indicates the nuclei of cells. Magnification 40x.

### **5.6. Enzymatic muscle single fiber dissociation and free myoplasmic $\text{Ca}^{2+}$ concentration ( $[\text{Ca}^{2+}]_i$ ) measurement**

Enzymatically dissociated flexor digitorum brevis (FDB) muscle fibers were used in some of our studies to detect the effects of TLR ligands on muscles. The protocol was described previously by Liu and co-workers [148]. Briefly, the whole FDB muscles were removed from the toe of the mice. The tendons, connective tissue and blood vessels around FDB muscles were cleaned carefully under a microscope using a pair of forceps and micro-iris scissors. Clean FDB muscles were incubated for 2-3 hours at 37 °C in 0.3% collagenase type I in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Then muscles were transferred to fresh DMEM and gently triturated about 30 times to dissociate single muscle fibers. 300  $\mu\text{L}$  of the resultant muscle fiber suspension was then transferred to laminin coated glass-bottom Petri dishes and allowed to attach for up to 15 minutes before 2.7 ml DMEM supplemented with antibiotic, antimycotic solution (1  $\mu\text{L}/\text{ml}$ , Sigma) was added. Fibers were incubated overnight at 37 °C for use on the following day.

$[\text{Ca}^{2+}]_i$  was measured in the dissociated fibers during a fatiguing protocol consisting of 300 intermittent tetanic contractions (70 Hz, 350 ms duration, 2 s intervals) using the fluorescent  $\text{Ca}^{2+}$  indicator indo-1. Briefly, muscle fibers attached to laminin-coated glass-bottom dishes were incubated with indo-1 AM (4  $\mu\text{M}$ ) for 20 min, followed by a 20 min wash. Change in indo-1 fluorescence was measured with a PTI system consisting of a Xenon lamp, a monochromator and two photomultiplier tubes. Excitation light was  $360 \pm 5$  nm and the emitted fluorescence was collected after passing through a dichroic mirror at



405 ± 5 nm and 495 ± 5 nm. The ratio of the light emitted at 405 nm and 495 nm was translated to  $[Ca^{2+}]_i$  as described previously [149, 150].

### **5.7. Mechanical muscle single fiber dissection, force and $[Ca^{2+}]_i$ measurement**

Mechanically dissected single fibers were used to measure muscle force and  $[Ca^{2+}]_i$  as described earlier [151-153]. Briefly, single fibers were isolated under a dark-field illumination microscope using a pair of forceps and micro-iris scissors. The FDB muscles were split into three bundles after which each bundle was trimmed down to two or three fibers. One fiber was selected and the rest were killed by cutting through the sarcolemma, leaving only the selected fiber alive. The fiber's ability to contract was tested by focal electrical stimulation. After isolation of a single fiber, the tendons were gripped by platinum-foil micro-clips and the preparation was transferred to the perfusion channel of an experimental chamber, placed on the stage of an inverted microscope. The bottom of the chamber consisted of a thin glass cover slip and fibers were mounted as close as possible to this. The fiber was flanked by platinum electrodes which were used for stimulation.

Force from mechanically dissected fibers was measured using an Akers 801 force transducer vertically mounted in the experimental chamber. The force transducer was provided with a glass tube extension with a fine platinum hook at the end. On the other end of the chamber, there was an adjustable hook. The tendon clips were attached to the two hooks, suspending the fiber horizontally. The fiber was stretched to the optimal length (the length giving the maximal tetanic force) and the diameter of the fiber at this length was measured in order to calculate the developed force per cross-sectional area.

The  $[Ca^{2+}]_i$  measurements in these dissected fibers are basically same as in the enzymatically dissociated fibers as described above. The main difference was that the dissected fibers were loaded with indo-1 by pressure-injection into the fibers. Subsequent procedures to measure  $[Ca^{2+}]_i$  were the same as described earlier for the dissected fibers.

### **5.8. Western blot**

For western blot, the muscles were homogenized with a motor-driven ground glass homogenizer in ice cold homogenization buffer (20 µl per mg wet weight; pH 7.4) The homogenate was centrifuged at 700g for 5 min at +4°C. The supernatant was transferred to a new tube and the protein concentration was determined using the Bradford assay. Lysates were diluted 1:1 in Laemmli buffer, left overnight at +4 °C and then heated for 10 min at +70 °C. Subsequently, 10 µg of protein was loaded and run on a NuPAGE Novex 4-12% precast Bis-Tris gel. Proteins were then transferred to polyvinylidene fluoride membranes. Membranes were blocked for 1 hour at room temperature in blocking buffer, followed by incubation overnight at +4 °C with primary antibodies. Membranes were washed in Tris-buffered saline-Tween 20 (TBS-T) and incubated for 1 hour at room temperature with

secondary antibody. Membranes were then washed in TBS-T and immunoreactive bands were visualized by an infrared fluorescence microscopy.

## **6. Results and discussion**

In the following sections the main results of Paper I-IV are summarized and together with unpublished data discussed in the context of recent findings and literature.

### **6.1. Paper I: Anakinra treatment in patients with refractory inflammatory myopathies and possible predictive response biomarkers: a mechanistic study with 12 months follow-up.**

Overexpression of IL-1 $\alpha$  and IL-1 $\beta$  was demonstrated in muscle tissue of patients with myositis [50, 51], which indicate a role of IL-1 in the pathogenesis of myositis. Moreover, some beneficial effects of IL-1 blockade were reported in a case study of one myositis patient with anti-Jo-1 autoantibodies [52], but whether IL-1 has a role in the pathogenesis was not clarified. Therefore, we initiated a mechanistic, open label study with anakinra in fifteen patients with refractory myositis.

We found that seven out of fifteen patients with refractory myositis responded to anakinra according to the IMACS definition of improvement. Furthermore, we showed that clinically, patients with extra-muscular symptoms responded better to anakinra than those without; immunologically, CD163<sup>+</sup> macrophages, IL-1 $\alpha$  expression in muscles, and CD4<sup>+</sup> activated memory T cells in blood might be negative markers for response to anakinra treatment. Due to the limited number of the patients in this study these results should be concluded carefully. Finally, we demonstrated that neutralizing IL-1 reduced T cell differentiation into Th17. Immunohistochemical analyses did not show any differences in inflammatory cells or molecules including T cells and IL-1 in the muscles before and after treatment.

In conclusion, anakinra treatment could be beneficial in a subgroup of patients with myositis, but biomarkers to predict response need further investigation. The lack of effect on IL-1 expression in muscles questions the role of IL-1 in the mechanisms that cause muscle weakness in these patients. Our data also suggest that a large scale placebo controlled trial with anakinra in myositis patients is needed. Such an investigation may also be able to shed more light on the molecular mechanisms of anakinra and on the role of IL-1 in disease mechanisms in myositis.

### **6.2. Paper II: Effects of immunosuppressive treatment on IL-15 and IL-15R $\alpha$ expression in muscle tissue of patients with polymyositis and dermatomyositis.**

In our anakinra study, we found that T cells persist in muscle tissue after treatment, even in the patients who have good response to anakinra. Our earlier observation also showed that while the number of macrophages are decreased or even disappeared after glucocorticoid treatment, T cells in muscle tissue are usually maintained [53, 54]. As the most important T cell-growth factor, IL-2 is rarely found in muscle tissue of patients with myositis, it is

possible that other cytokine (s) may have IL-2-like functions stimulating T cell proliferation and differentiation [55-59]. In this context, we started our IL-15 investigation. The study was based on muscle biopsies before and after immunosuppressive treatment in seventeen recently diagnosed patients.

We found that IL-15 was expressed in macrophages in muscle tissue of all patients with polymyositis or dermatomyositis and IL-15R $\alpha$  was expressed in inflammatory cells in 15 patients, on capillaries of 11 patients and large vessels of 13 patients. Only occasional mononuclear cells were found to be positive for IL-15 and IL-15R $\alpha$  in healthy controls. An inverse correlation was found between baseline IL-15 expression in muscle tissue of patients and changes of the muscle performance  $\Delta$ FI ( $r=-0.81$ ,  $P=0.0002$ ). There was a reduction in the number of IL-15 and IL-15R $\alpha$  positive cells after a median of eight months with conventional immunosuppressive treatment, but nearly half of the patients (8 out of 17) still had a higher number of IL-15 expressing cells in muscle tissue after treatment compared to healthy individuals and this group of patients had less improvement in muscle performance test compared to those patients with a low number of IL-15 expressing cells in the muscles after treatment, both after eight months treatment and at a five-years follow up.

A correlation was found between baseline IL-15 expression and CD3+ T cells in muscle tissue of patients ( $r=0.6$ ,  $p=0.02$ ). And the change of IL-15 expression after treatment correlated with change of CD3+T cells ( $r=0.6$ ,  $p=0.01$ ). Immunofluorescence demonstrated a close localization between IL-15 positive cells and CD3+T cells in muscle tissue of patients. Thus, the positive correlation between IL-15 and CD3+T cells, and their close localization might support our hypothesis that IL-15 might help to sustain T cell activation and homeostasis in muscle tissue of patients with myositis [65, 154, 155].

In conclusion, we demonstrated that IL-15 and IL-15R $\alpha$  are expressed in muscle tissue of patients with PM or DM. They might play a role in sustaining T cell proliferation and homeostasis in muscles. Moreover, a correlation was found between IL-15 expression and clinical outcome. Therefore, targeting IL-15 might be a novel potential therapeutic target in PM and DM, at least in that subgroup of patients with high IL-15 expression in muscle tissue after conventional immunosuppressive treatment.

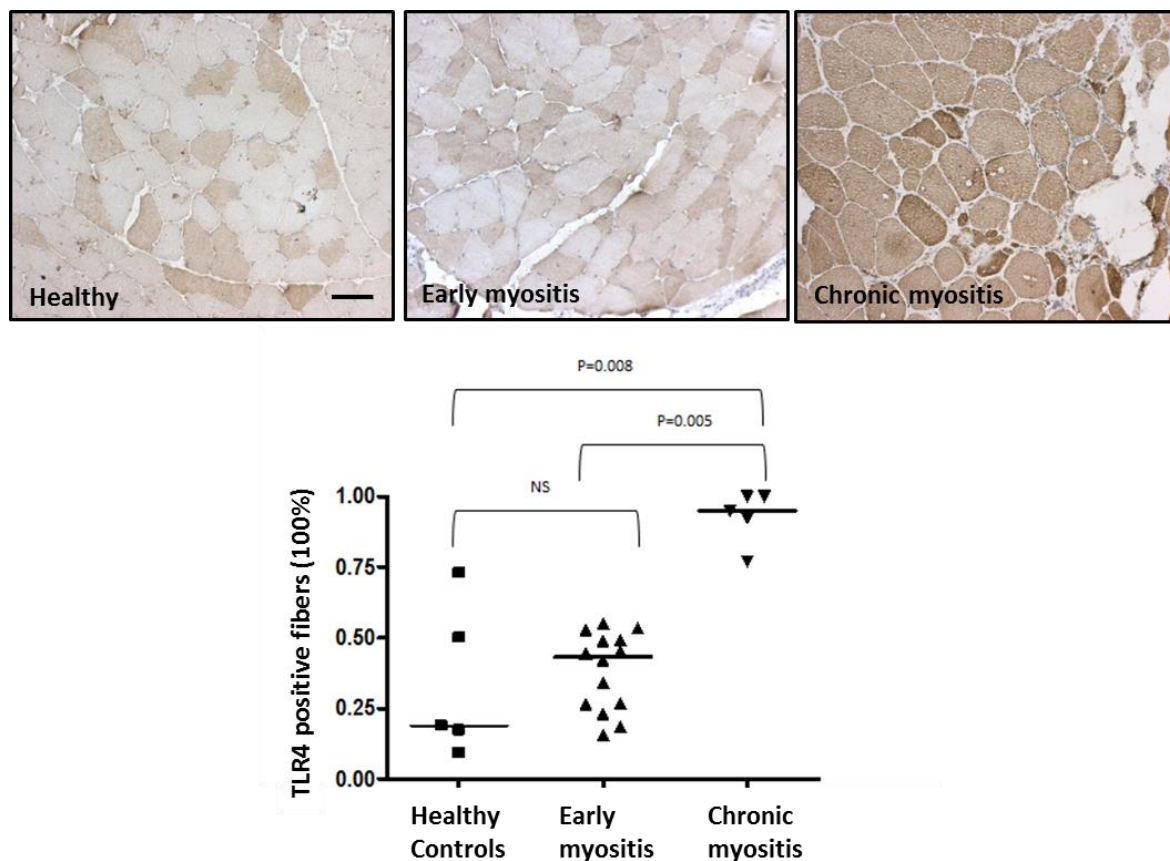
### **6.3. Paper III: TLR4 as receptor for HMGB1 induced muscle dysfunction in myositis.**

Next, we focused on the question on which other mechanisms could contribute to muscle weakness than T cell mediated myocytotoxicity. To address this question we selected to investigate muscle biopsies from a group of patients with muscle weakness and muscle fatigue but whose muscle biopsies had no obvious inflammatory infiltrates. Earlier we had observed that myoplasmic localization of the inflammatory mediator HMGB1 is often found in combination with MHC-class I in muscle fibers from patients with myositis even

when inflammatory infiltrates were not detectable. Furthermore, exogenous HMGB1 could accelerate the development of muscle fatigue and induce MHC-class I expression in adult mouse skeletal muscle fibers *in vitro* [9, 10]. In order to investigate the mechanisms by which HMGB1 may worsen muscle fatigue, we set out to investigate which receptors HMGB1 acts via in adult skeletal muscle fibers in order to contribute to muscle fatigability and MHC-class I expression. We used dissociated single muscle fibers from FDB of WT, RAGE knock-out (RAGE<sup>-/-</sup>) and TLR4 knock-out (TLR4<sup>-/-</sup>) mice. Immunohistochemistry was used to investigate TLR4, MHC-class I and myosin heavy chain expression in muscle fibers from fourteen patients with myositis with a short duration of muscle weakness and without detectable cellular infiltrates in muscle biopsies.

From the animal study we found that TLR4, but not RAGE, may mediate muscle fiber dysfunction via HMGB1. In myositis patients, we demonstrated that TLR4 was expressed on muscle fibers. By staining of consecutive sections of muscle biopsies from myositis patients for TLR4 and MHC-class I expression we could demonstrate that these two molecules were co-localized to the same muscle fibers and that these fibers were fast twitch type II fibers. As there was no inflammation or other pathological changes apart from cytosolic localization of HMGB1 in the muscle fibers of these patients, we speculate that HMGB1 may act via TLR4 to induce expression of MHC-class I. This hypothesis is supported by the results from our *in vitro* study of isolated mouse muscle fibers. Furthermore, we also established that as the disease progresses, both MHC-class I and TLR4 could be detected in an increasing number of muscle fibers [47] (Figure 3). We also detected TLR4 expression in muscle fibers of healthy individuals but HMGB1 and MHC-class I were absent which suggested again that HMGB1 plays a key role in causing muscle dysfunction in patients with myositis.

In conclusion, exposure of differentiated skeletal muscle fibers to HMGB1 resulted in greater muscle fatigue and induced MHC-class I expression via a TLR4-dependent pathway. HMGB1, TLR4 and MHC-class I co-localization was frequently observed in muscle fibers of myositis patients. Therefore, the HMGB1-TLR4 pathway is a potential novel target for therapy in myositis patients. This may in particular be interesting in the chronic phase, when treatment with conventional therapies has failed.



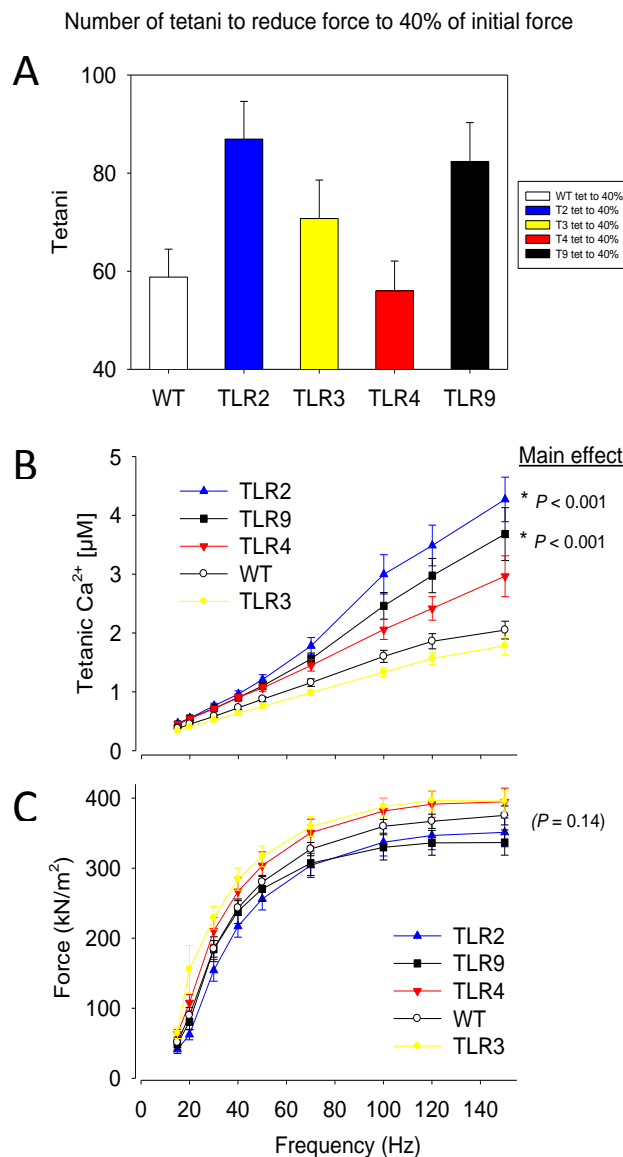
**Figure 3.** Representative pictures of TLR4 immunohistochemistry staining in muscle biopsies from healthy individuals, early myositis and chronic myositis patients. Early myositis group consisted of 14 patients (8 females and 6 males, median age 46 years, range 26-61) with short duration of clinical symptoms (median 4 months, range 1-9) and without detectable cellular infiltrates in muscle biopsies. Chronic myositis group consisted of five patients (3 females and 2 males, median age 66 years, range 53-76) with a long duration of clinical symptoms (median 55 months, range 48-72) and cellular infiltrates in their muscle biopsies. Healthy individuals (4 females and 1 males, median 63 years, range 50-78) without any clinical or histopathological signs of muscle disease were used as controls. Brown color shows the positive staining. Nuclei stained by hematoxylin were shown by blue color. Scale bar 25µm. Magnification x10.

#### 6.4. Paper IV: Loss of TLR2 increases fatigue resistance in mouse skeletal muscles.

Apart from RAGE, TLRs including TLR2 and TLR4 are also receptors that HMGB1 may use to bind and transfer signals. During our exploration in paper III, we found some interesting muscle function modifications when TLR2 was knocked out. Therefore we decided to further investigate this molecule and its relation to muscle fiber weakness.

TLRs, a group of receptors that bind the peptides from bacteria/virus, are expressed on immune cells and contribute to the innate immune response. Recently several studies have shown that TLRs can also be expressed on non-immune cells including skeletal muscle fibers. However, the functions of TLRs on these non-immune cells are poorly understood.

When we explored the receptors through which HMGB1 may exert its effects on muscle fibers we used knock-out mice for the receptors known to mediate effects of HMGB1, and observed that muscle fibers from TLR2 knock-out mice displayed different properties upon stimulation compared to fibers from WT and other TLR knock out mice. The fibers from TLR2<sup>-/-</sup> mice demonstrated significantly increased fatigue-resistance as shown by the number of tetani needed to reduce force to 40% of the initial force when fatigued with repeated intermittent tetanic stimulation (Figure 4A). We also observed that tetanic  $[Ca^{2+}]_i$  was statistically significantly higher at all stimulation frequencies ( $p < 0.001$ ) in TLR2<sup>-/-</sup> compared to WT muscle (Figure 4B), while tetanic force was similar in TLR2<sup>-/-</sup> and WT muscles (Figure 4C). A similar improved muscle fatigue-resistance was observed in TLR9<sup>-/-</sup> mice and a trend in TLR3<sup>-/-</sup> mice, but their tetanic  $[Ca^{2+}]_i$  was not changed. Therefore, we focused our further investigations on the roles of TLR2 in skeletal muscle function.



**Figure 4.** Skeletal muscle fibers from TLR2<sup>-/-</sup> mice showed increased fatigue-resistance and more tetanic  $[Ca^{2+}]_i$ . (A) Number of tetani needed to reduce force to 40% force is greater in TLR2 than in WT dissected single FDB muscle fibres. A similar improved muscle fatigue-resistance was also observed in TLR9<sup>-/-</sup> mice and a trend in TLR3<sup>-/-</sup> mice, but not in TLR4<sup>-/-</sup> mice. (B) Tetanic  $[Ca^{2+}]_i$  was statistically significantly higher in TLR2<sup>-/-</sup> compared to WT muscle but this property was not observed in TLR4<sup>-/-</sup>, TLR3<sup>-/-</sup> and TLR9<sup>-/-</sup> mice. (C) Tetanic force at all stimulation frequencies was not different between TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>, TLR3<sup>-/-</sup>, TLR9<sup>-/-</sup> and WT muscles.

We next stimulated the muscle fibers from WT mice with known TLR2 ligands, HMGB1 and Pam3CSK, and we found that activation of TLR2 signals with either ligand could accelerate muscle fatigability. Western blots were also performed to investigate the muscle intrinsic proteins and mitochondrial enzyme activities in muscles from WT and TLR2<sup>-/-</sup> mice. We found that enhanced fatigue-resistance in TLR2<sup>-/-</sup> mice could relate to the increased anti-oxidant ability as superoxide dismutase (SOD) 1 and SOD2 were increased dramatically in these knock out mice. Moreover, the higher tetanic calcium release from TLR2<sup>-/-</sup> mice could be explained by an increased amount of calcium-binding proteins in the muscles as more calsequestrin and sarcalumenin were found in these mice. Next we investigated whether TLR2 is expressed in skeletal muscle biopsies from patients with myositis. Indeed, we found that TLR2 together with one of its endogenous ligand, HMGB1, was expressed in muscle fibers of patients with myositis.

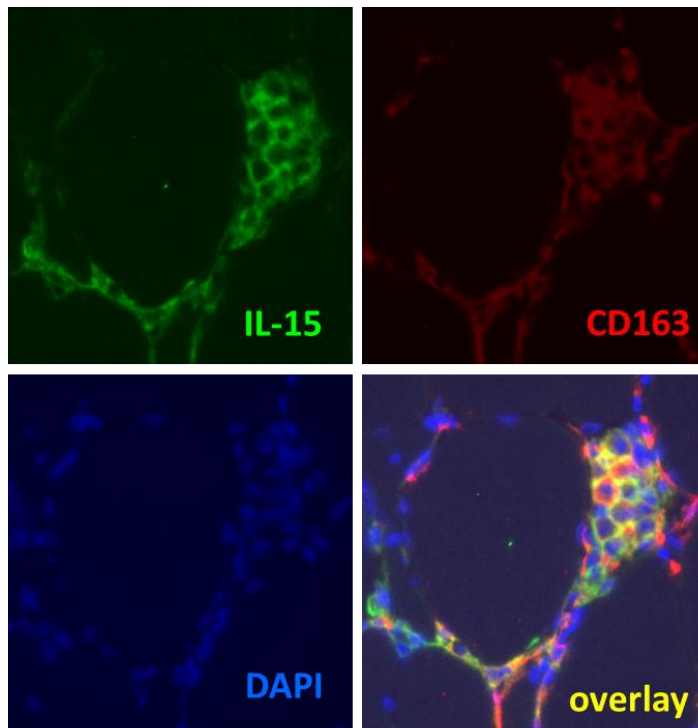
Together with the animal study, we suggest that the TLR2 signal could play a role in causing muscle problems in patients with myositis and modifying this signal may be a way to improve muscle function of these patients in the future.



## 7. Future perspectives

**IL-1:** Whether IL-1 has a role in the pathogenesis in myositis is still unclear. Blocking IL-1 by anakinra had beneficial effects on clinical outcome measure in some patients with refractory myositis. An indication of a biological effect was suggested by the effects on peripheral blood cells but no effect was seen on the inflammation in muscle tissue. From our mechanistic study with a limited number of patients we could not identify predictive markers for responders in whom IL-1 would be more likely to have a role in the molecular pathways of disease. This would require a larger controlled trial. Such a trial is ongoing in collaboration with a pharmaceutical company.

**IL-15:** In our first study we investigated IL-15 and its specific receptor expression in patients with PM and DM. Now I have completed staining on a few patients with IBM and could demonstrate that IL-15 is also expressed in muscle tissue of this subgroup of patients (Figure 5). In the future, we should confirm their expression in IBM in a larger cohort.



**Figure 5.** IL-15 was expressed in CD163+ macrophages in muscle tissue of patients with IBM. (A) Immunofluorescence staining showing IL-15 expression in inflammatory cells. Green color indicates positive staining. (B) CD163+ macrophages in red by immunofluorescence. (C) Nuclei were shown by DAPI. (D) Overlay picture demonstrates that IL-15 was expressed by CD163+ macrophages. Magnification 32x.

In addition, T cell infiltration in muscle tissues of myositis patients is an important histological feature of these diseases, especially in PM and IBM patients. We have previously shown that T cell infiltrates in muscle tissue of PM and DM patients are

dominated by a subset of T cells so called CD28<sup>null</sup> T cells, and these infiltrates often persist even after immunosuppressive treatment which indicate some continuous stimulator for these T cells [156]. CD28<sup>null</sup> T cells were also shown to be expanded in blood of myositis patients. In addition, muscle and blood CD28<sup>null</sup> T cells shared the same restricted TCR-V $\beta$ , suggesting that these cells may have a common origin and antigen specificity [157]. One important function of IL-15 is to stimulate proliferation of human memory CD8<sup>+</sup>T cells. This has been proven by IL-15<sup>-/-</sup> mice in which reduced numbers of memory-phenotype CD8<sup>+</sup> T cells were found in the spleen and lymph nodes that were reversible upon provision of exogenous IL-15 [57]. Interestingly, a role for common gamma chain cytokines in the down-regulation of CD28 expression on CD8<sup>+</sup> T cells has been demonstrated. As an important member of these cytokines, IL-15 has been shown to play an important role in the generation of CD28<sup>null</sup> CD8<sup>+</sup> memory T cells [158]. In our studies we have now demonstrated that both IL-15 and CD28<sup>null</sup> T cells exist in the muscle tissue of myositis patients. In the future it might be interesting to determine the role that IL-15 plays in the generation and proliferation of CD28<sup>null</sup> cells of either CD4 or CD8 lineage in myositis patients.

**HMGB1:** HMGB1 has from our studies turned out to be a potential candidate to block in future studies of patients with myositis. In particular, HMGB1 may have a role in the early phase of the disease as a factor triggering the initial local inflammation in the muscle tissue having effects on muscle fiber phenotype with e.g. upregulation of MHC class-I and also clearly having effects on muscle fiber performance inducing muscle fatigue. At the cellular level, the signaling pathways by which HMGB1 impairs muscle performance require further investigation. The redox states of HMGB1 in myositis and whether cytokine signatures can modify its redox states also need detailed future studies. In addition, one interesting demonstration in our immunohistochemistry study is that both HMGB1 and TLR4 (also TLR2) can be detected in the cytosol of muscle fibers, which raises the possibility that they could bind to each other and mediate muscle dysfunction before they are released out of cells. This hypothesis needs to be further investigated. Finally, the mechanism (s) that causes HMGB1 to translocate from nuclei to cytosol especially in muscle fibers from patients with normal-looking muscle morphology and without inflammatory infiltrates is still unclear and should be investigated. In this context the role of hypoxia is interesting. Hypoxia has been demonstrated to induce HMGB1 release from hepatocytes [159], in synovitis [160] and in ischemia-reperfusion injuries [161, 162]. Hypoxia has been implicated to occur in myositis. Capillaries are lost in muscles of patients with PM and DM [163, 164]. VEGF is highly expressed in muscle tissues [165]. Furthermore, hypoxia-inducible 1- $\alpha$ , a sensitive marker of hypoxia, is overexpressed in muscle tissues of patients with PM and DM compared with healthy individuals [166]. In addition, hypoxia can also prevent cysteines from being irreversibly oxidized and therefore potentially provides a supportive environment for HMGB1 to stay in the dangerous redox

state enabling its binding to TLR4 [167]. Muscle is an organ that is particularly dependent on oxygen for its metabolism and thus sensitive to hypoxia, whether hypoxia in muscle tissue could be a trigger of inflammation that in some individuals lead to a chronic inflammation still needs to be investigated.

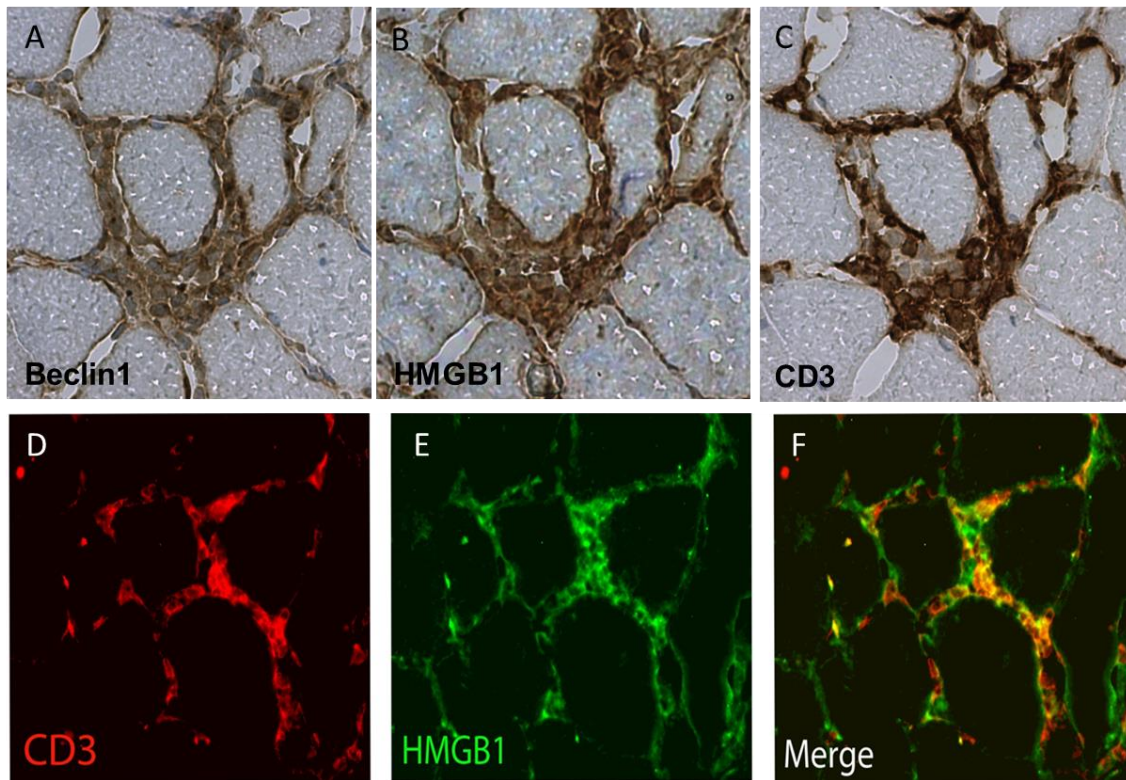
**TLRs:** We believe that our current study just reveals a tiny tip of the iceberg about the roles of TLRs in the skeletal muscle function. It will be very interesting to determine other TLRs expression and function in the muscle fibers of patients. For example, TLR7, TLR8 and TLR9 are related to the type I interferon signaling which has been shown to be expressed in muscle tissue and peripheral blood of subsets of myositis patients especially in dermatomyositis patients and in patients with anti-Jo-1 antibodies. Therefore, it would be interesting to investigate the expression of these TLRs and to see how muscle function will be influenced by modifying their expression in skeletal muscle using mouse models. There are hints that TLRs expression is altered by the health status. It was already shown that exercise can reduce TLR2 and TLR4 expression on monocytes in healthy individuals [168, 169]. Exercise can benefit myositis patients with improved muscle performance but the mechanism underlying this effect still needs to be clarified [41, 43, 170-174] . The TLRs and how they change after exercise in myositis patients would be interesting to explore in future studies.

**Beclin1:** In addition to the above inflammatory cytokines/molecules that we investigated, during my PhD study I also became very interested in another molecule, Beclin1, which is a molecule involved in the upstream autophagy signaling.

A recent study showed that endogenous, cytosolic HMGB1 protein can induce autophagy by binding Beclin1 (an upstream protein initiating autophagy) and help the cell to survive under certain conditions [175]. This raised our interest because we have found that HMGB1 is abundantly expressed in the cytosol of inflammatory cells in muscle tissue of patients with myositis [10]. T cells express genes encoding autophagy which seems to be essential for proliferation of T cells after receptor activation [176]. Thus, there is a possibility that HMGB1 may also contribute to the T cell survival and proliferation in myositis by binding Beclin1 and inducing autophagy and thereby promote the chronic inflammation in muscle tissue. Therefore, we investigated whether Beclin1 is expressed in the muscle tissue of patients with myositis.

We found that both Beclin1 and HMGB1 were detected in mononuclear infiltrating cells in muscle tissue of nine investigated patients with myositis, and their expression co-localized to the infiltrating T cells in muscles as demonstrated by both staining of consecutive sections (Figure 7A-C) and double fluorescence staining of muscle tissue (Figure 7D-F). Therefore, we suggest that autophagy of T cells could be initiated by HMGB1-Beclin1

binding in muscle tissue of patients with myositis and this pathway may contribute to T cell survival in the muscles. This project is now under investigation.



**Figure 6.** Beclin1 and HMGB1 were expressed in muscle tissue of patients with myositis and co-localized to infiltrating T cells. (A) A representative section of Beclin1 expression in the infiltrating cells in the muscles. Brown color indicates positive staining. Blue color: nuclei of the cells. Note that bluish nuclei can be seen clearly in the infiltrating cells which indicate that Beclin1 was mainly localized to the cytosol. (B) A consecutive section stained with HMGB1 showed that HMGB1 was expressed in the infiltrating cells. Note that bluish nuclei are not easily visible anymore which indicates that HMGB1 localized to both the nuclei and cytosol of infiltrating cells. (C) A consecutive section stained by CD3 marker showing that T cells dominated in the infiltrates. (D) Immunofluorescence staining showing CD3+ T cells in the infiltrates. Red color indicates positive staining. (E) Immunofluorescence staining indicated HMGB1 expression in the infiltrating cells. Green color indicates positive staining. (F) Overlay picture of D and E showing that HMGB1 is expressed in the T cells; Magnification 20x in A-C and 10x in D-E.

## 8. Conclusions

Myositis is a group of heterogeneous diseases primarily with muscle weakness and muscle fatigue as the most common and dominant symptoms. High-dosage of long-term glucocorticoids is the most widely used treatment so far, but efficacy is limited and side effects are worrying. Therefore, development of new therapies is strongly needed. Biological therapies, e.g. TNF blockade, have been encouraging in RA. However, the resistance to these therapies in myositis emphasizes the need for fundamental molecular knowledge to help develop new treatment options for patients with myositis.

In my thesis, we investigated the expression of four molecules in muscle tissue and their potential roles in the disease development of myositis. In brief, we found that: **(I)** IL-1 may have a role in the disease mechanisms in a subgroup of patients but this needs to be further explored. **(II)** IL-15 may be a potential new target for treatment as IL-15 and IL-15R $\alpha$  were expressed in muscle tissue of patients with myositis even after immunosuppressive treatment and patients with higher levels of IL-15 had a more severe muscle dysfunction both from a short and long term observation. **(III)** HMGB1 and TLR4 are present in muscle tissue of patients with myositis who have muscle weakness and *in vitro* experiments showed that rHMGB1 can accelerate muscle fatigue and induce MHC-class I via TLR4. Therefore the HMGB1-TLR4 pathway may be of interest for future targeting therapies in these patients. **(IV)** Finally, TLR2, a receptor for pathogens in immune cells may have a totally different role in muscle fibers and may be involved in the mechanism of muscle fatigue; the explanation for this is unclear but raises the possibility of a new pathway to target in future studies in patients with muscle weakness and fatigue.

In summary, the investigations of the studies in my thesis suggest that different molecules may play a role in the pathogenesis of myositis. Different molecules can contribute to the disease development at different stages and through different mechanisms. We did not find one key molecule in myositis at least not during my explorative journey, and it is hard to believe in its existence due to the complexity and heterogeneity of myositis. A possible way to continue the molecular studies in myositis in the future might be in subgroups with homogenous clinical phenotypes and should involve a combined investigation of genes and environment as well as in combination with animal models.

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**To my family.**



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